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The role of transforming growth factor-β signalling in the patterning of the dentary of the mouse

Neal Anthwal, Department of Craniofacial Development, Kings College London
Yang Chai, Centre for Craniofacial Molecular Biology, School of Dentistry, University of Southern California
Abigail S Tucker, Department of Craniofacial Development, Kings College London.

Address for correspondence:
Department of Craniofacial Development, 27th Floor, Guy's Tower, Kings College London, London SE1 9RT
Tel +44(0)20 7188 7389, +44(0)20 7184, +44(0)20 7188 1795,
Fax +44(0)20 7188 1674

Abstract

The evolution of the novel mammalian jaw articulation, the squamosal-dentary or temporomandibular joint, has resulted in an increased complexity and modularity of the dentary bone, reflecting the multiple roles it now fulfils as the primary bone of the mandible. In mice this patterning, with three proximal processes, is apparent by expression of *Runx2* before the bone ossifies. Following ossification the angular and condylar processes grow with the addition of secondary cartilages under the control of chemical and mechanical signals. Previous studies indicate that signalling though Tgfbr2, the type II receptor of Tgf-β signalling, is important in the development and patterning of the proximal articular portion of the dentary, and of the angular process in particular. We show that the expression of the *Tgf-β 2* isoform is associated with the developing angular process, and that the connective tissue marker *Scleraxis* is co-expressed with, and inducible by, and down regulated by the inhibition of, *Tgf-β* in this region, suggesting a role of mechanical force and Tgf-β signalling in the development of the angular process. We also demonstrate that the secondary cartilages are inducible in explant culture in the absence of mechanical stimulation, and that this induction can be prevented by inhibition of Tgf-β signalling. This study suggests that the developing mouse mandible is able to induce secondary cartilage in the absence of mechanical force through the Tgf-β signalling pathway, but the proper development of the processes and their cartilages in general and the angular process in particular is reliant on both mechanical forces and Tgf-β signalling working in concert.

Introduction

The lower jaw, or mandible, performs multiple functions: it forms the jaw joint with the upper jaw, carries the dentition and serves as an attachment site for the muscles of mastication. In non-mammalian quadrupeds the mandible is a compound structure made up of a number of membranous and endochondrally derived bones. In mammals, however, a single membranous bone, the dentary, serves these varied functions. The multiple functions of the mammalian dentary is reflected in its division into a number of different morphological and functional units (Klingenberg et al., 2003). Most commonly the units described are: the mandibular body, the alveolar bones of the molar and incisor teeth, the mental/rostral process, or "chin", and the three proximal processes, the coronoid, condylar and angular (Atchley and Hall, 1991; Atchley et al., 1985). Using a morphometric approach, it has been demonstrated that variation between each unit is greater than that within, suggesting that they are indeed separate units (Klingenberg et al., 2003). Recent mouse knockout studies appear to support this view. For example a neural crest specific loss of *Alk2*, a type I Bmp receptor, results in a loss of the rostral process and the secondary cartilages, but leaves the mandibular body relatively untouched. Similarly, loss of the transcription factors *Pax9*, *Dlx5*, *Tbx1* and a double knock out of *Prx1* and *Prx2* each results in the loss of the coronoid process, and not the angular or condylar processes (Depew et al., 1999; Jerome and Papaioannou, 2001; Peters et al., 1998; ten Berge et al., 1998). The developing mandible can also be viewed in terms of a series of nested developmental units: Depew and others suggest that each portion of the first branchial arch, the mandible and its counter part in the maxilla, is divided into proximal to distal units about the hinge, due to a nested pattern of *Dlx* transcription factor expression (Depew et al., 2005).

The posterior processes of the mandible are capped with secondary cartilages, the function of which is to facilitate growth and to enable the articulation of the dentary with the cranial base at the squamosal (or squamosal portion of the temporal) bone (figure 1 E) (Beresford, 1981; Depew et al., 2002; Frommer, 1964). This morphology, and the manner of its articulation with the cranial base, is one of the defining criteria of mammals and so when coupled with the knowledge that a number of genetic

disorders include mandibular abnormalities (Jakobsen et al., 2007; Mueller and Callanan, 2007; Nezarati and Aftimos, 2007; Suri et al., 2006), the importance of the study of the development of the mandible becomes apparent.

The gnathastome mandible develops from the first branchial arch, the mesenchyme of which includes a population of cranial neural crest cells (CNCs) (Noden, 1983). In the mouse a significant proportion of the skeletal and cartilaginous tissue of the mandible, including the squamosal-dentary joint, is derived from ectomesenchymal cranial neural crest cells (Chai et al., 2000). Membranous bones, such as the flat bones of the cranial vault and the body of the dentary, ossify directly from the mesenchyme; this differs from the majority of bones in the rest of the body, such as the long bones and the bones which form the jaw articulation in non-mammalian tetrapods, which ossify from a cartilage template by the process of endochondral ossification. The mammalian mandible does however posses some elements which undergo endochondral ossification, for example the secondary cartilages of the condylar and angular processes, and the posterior portion of Meckel's cartilage, which forms the malleus and incus of the middle ear (Amin and Tucker, 2006; Frommer, 1964; Silbermann and Frommer, 1972; Vinkka, 1982).

While it is established that the secondary cartilages of the mandible facilitate growth of the processes and, in the case of the cartilage of the condylar process, aide in the articulation of the mandible with the cranial base (Beresford, 1981), the exact nature of their development is still in debate. The secondary cartilages have been reported to grow in contradictory manners in closely related species, for in the mouse they develop continuous with the bony process, whilst in the rat, they seem to develop as a sesamoid, apparently distinct from the developing bone (Vinkka, 1982; Vinkka-Puhakka and Thesleff, 1993).

A number of molecules have been demonstrated to be important in the development and patterning of the mandible, one group of which being the Tgf-βs. The three Transforming growth factor β (Tgf-β) isoforms, Tgf-β1,2 and 3 represents a three member group of the Tgf-β superfamily of signalling molecules; this superfamily contains more than 30 members, including the Tgf-βs, Bone Morphogenetic Proteins (Bmps) and activins, and to important for normal craniofacial development (Dudas and Kaartinen, 2005). *In vivo* and *in vitro* investigations have demonstrated the

importance of Tgf-β in chondrogenesis and osteogenesis (Alvarez et al., 2002; Janssens et al., 2005; Mukherjee et al., 2005). Knockout studies of *Tgf-β 1, 2* and *3* demonstrate that the phenotype of *Tgf-β 2 -/-* mice have a number of unique phenotypes. These include hypoplasia of the mandible, including a loss of the angular process, diminished condylar and coronoid processes, but an apparent retention of the secondary cartilages (Sanford et al., 1997). This suggests an important role of Tgf-β 2 in the development of the proximal portion of the dentary. Targeted deletion of *Tgfbr2*, the common type II receptor for all three isoforms of Tgf-β (Kitisin et al., 2007), in the *wnt1* expressing neural crest cells using cre recombinase system (*Tgfbr2 wnt1-cre fl/fl*) produces a similar phenotype in the dentary, although in this case the secondary cartilage on the angular fails to form and the condylar cartilage fails to develop mature chondrocytes or undergo ossification (Ito et al., 2003; Oka et al., 2007).

Previous work used the *Tgfbr2 wnt1-cre fl/fl* mouse to investigate the role of Tgf-β signalling in chondrogenesis and osteogenesis during mandible development (Oka et al., 2007). In this study it was found that the mandible hypoplasia observed in these mice was not due to a failure in migration of the neural crest cells into the 1st branchial arch but due to a reduction in proliferation in Meckel's cartilage and the presumptive dentary mesenchyme. *Msx1*, a potential Tgf-β controlled inducer of osteoprogenitor cell proliferation, was diminished in the undifferentiated mesenchyme of the dentary.

Whilst the above study revealed the role of Tgf-β on the proliferation and differentiation of the cartilages of the mandible, its role in patterning the mandible was not addressed. In light of this, we aim here to investigate the role of Tgf-β signalling in patterning the mandible, specifically the three proximal processes. We also investigate the role of mechanical forces, acting through developing muscles and their connective tissues, in growth of the angular process of the dentary and how Tgf-β signalling may interact with such forces.

Methods and materials

Embryo collection and dissection

CD1 female mice were mated over night. Following a successful plug, 12 midday was considered E0.5, assuming that copulation had occurred the previous night. At the embryonic stages required, the mothers were sacrificed by cervical dislocation and the embryos in the uterus were colleted into sterile phosphobuffered-saline (PBS). Following a wash in fresh sterile PBS, the embryos were transferred to a clean 75mm petri-dish, removed from the uterus, membranes and placenta, and transferred to a petri-dish containing fresh PBS. Since the embryos were all over half of their gestation (=19-20 days), they were then killed by decapitation using a needle in accordance with UK law. Embryos were then processed for histology, wholemount analysis or explant culture.

Tissue processing and histological staining

Following fixation in 4% paraformaldehyde at 4ºC, wildtype and mutant embryonic mouse heads were dehydrated in an ethanol series and embedded in paraffin wax following clearing in Histoclear II. 8µm section were cut and serially laid out on superfrost plus slides. Mutant tissue was generated as in Oka et al (2007).

The first slide of each series was then rehydrated through ethanol and stained either with haematoxylin and eosin, or with sirrius red / alician blue using standard techniques. Subsequent slides in each series were used for *in situ* hybridisation.

In order to stain for alkaline phosphatase, samples were fixed in 70% ethanol and dehydrated to 100% in a graded series. Dehydrated samples were embedded in molten polyester wax at 42ºC. 10µm sections were cut using a conventional microtome. In order to ensure the wax remained hard the block was cooled at -20ºC before use and dry ice was used to cool the block and blade during sectioning. Sections were then laid out on to slides coated with 1% bovine albumin and 1% bovine gelatine (both Sigma) and left to dry over night at 32ºC. Following de-waxing in Histoclear II and rehydration through an ethanol series, sections were equilibrated in staining buffer made up of 0.15M Tris-HCl pH7.5, 4mM MgCl2 for 10 minutes. 100µl of staining

solution made up of 3.5µl BCIP and 4.5µl NBT per ml of buffer was added to each slide, which were coverslipped and left in the dark for 25 minutes at room temperature. The reaction was stopped in PBS, the slides dehydrated though ethanol and mounted in DePX mounting medium.

In situ hybridisation

Radioactive probes for mouse *Tgf-β 1, 2* and *3* (a gift from HL Moses), *Scx* and *Myf5* RNAs were made and *in situ* hybridisations were carried out to detect the expression of these genes in sagittal plain cut sections of e13.5-e15.5 wildtype and e14.5 *Tgfbr2 wnt1-cre fl/fl* mouse jaw joints using standard techniques (Mahmood and Mason, 1999). Expression of *Runx2* RNA was detected using a dig-labelled probe using standard techniques. Dig-labelled RNA probes for *Tgf-β 1* and *2* were generated for the use in whole mount *in situ* hybridisation, as previously described (Pelton et al., 1991).

Explant culture

In vivo explant cultures of embryonic tissue in both the mouse (Glasstone, 1971) and hamster (Vinkka-Puhakka and Thesleff, 1993) have reported development of mandibular secondary cartilages. Developing dentaries from mice of embryonic day 14.5 (e14.5) and e15.5 were cultured in a manner modified after that of Glasstone (Glasstone, 1971). Briefly, following the sacrifice of the mother, the branchial arches of e14.5 and e15.5 mice were dissected form the head, and with the aide of a dissecting microscope the dentary primordia isolated. The explants were then placed on micropore filters supported on steel grids and cultured in Biggers modified BGJ medium (BGJb) with the addition of penicillin/streptomycin, 10% foetal bovine serum and 100µg/ml ascorbic acid, at the air/medium interface. The medium was changed every second day, with the medium being made fresh each time. After 4 days in culture, the explants were fixed either in 95% ethanol for alizarin red and alcian blue staining, or 4% paraformaldehyde for histological analysis.

In order to establish the timing of the effect of Tgfbr2 signalling in the development of the dentary, explants of the developing dentary from e14.5 mice were cultured for 5 days with or without the addition of SB431542, a small molecule inhibitor of Alk 4, 5 and 7, the type I Tgf-β receptors phosphorlated by Tgfbr2, and an effective inhibitor of Tgf-β signalling (Inman et al., 2002). SB431542 dissolved as a stock of 10mM in

DMSO, 10µl of which was added per ml of modified BGJb, to give a final molarity of 10µM, in which the right hand side dentary of each embryo was cultured. The left hand dentaries were cultured as controls in BGJb with 10µl DMSO per ml of medium. Cultures were then fixed in 95% ethanol and processed for alician blue / alizarin red staining.

Additionally lower whole arch explants of e13.5 embryos were cultured as previously described (Tucker et al., 1998), but in the absence of serum to prevent any extra Tgf-β in the serum acting upon the cultures. In order to investigate whether Tgf-β is capable of inducing *Scx*, Affigel beads loaded with either Tgf-β 1 or Tgf-β 2 (R&D Systems) were then implanted lateral to Meckel's cartilage and outside of the endogenous region of *Scx* expression. Control beads loaded with BSA were also used. Additionally, e13.5 – e15.5 whole arch explants were cultured for 24 hours in serum free medium with the addition of either 10µM SB431542 or 10µl DMSO per ml of medium. These whole arch explants were then processes for *in situ* hybridisation.

Alizarin red – alcian blue staining

To observe the development of the skeletal and cartilaginous elements alizarin red – alcian blue double staining was carried out. The following method allows for differential staining of bone and cartilage with alizarin red and alcian blue respectively. e14.5 to p0 mouse embryos were fixed in 95% ethanol for 5 days. After 2 hours of fixation, the specimens of age e16.5 and above were skinned and eviscerated using fine forceps and the soft tissues discarded. Following fixation, the specimens were further dehydrated in by immersion in acetone for 2 days. The acetone was then removed and the embryos allowed to dry a little before being placed in a freshly made stain solution made from 1 volume 0.3% alcian blue 8GS in 70% ethanol, 1 volume 0.1% Alizarin Red S in 95% ethanol, 1 volume 100% glacial acetic acid and 17 volumes 70% ethanol for 3 days. The embryos were then washed briefly in distilled H2O then macerated in 1% potassium hydroxide (KOH) for 2 days, or until the bones and cartilages were clearly visible. The clearing was continues in increasing concentrations of glycerol in 1% KOH, for 1:3 through to 100% glycerol over 3 weeks. After analysis, the embryos were stored at 4ºC.

Results

Morphology of the developing mandible

In mouse development, the ossified dentary is first detectable by alizarin red at e14.5 as a thin element lateral to Meckel's cartilage. At this initial stage the dentary already has its characteristic three-processed form, with distinct coronoid, condylar and angular outgrowths. The condylar process secondary cartilage is first detected with whole mount alcian blue staining at e15.5, and the angular process cartilage is seen at e16.5 (figure 1 B-E).

In order to resolve the pattern of the developing dentary before ossification, alkaline phosphatase staining and *in situ* hybridisation for *Runx2* were carried out in sagittal sections of e13.5 mouse mandibles. *Runx2* is expressed in the mesenchyme of the future dentary in a form similar to that of the ossified element at e14.5, suggesting that the patterning of the dentary into three processes occurs early in development (figure 1 A). NBT/BCIP staining for alkaline phosphatase reveals a similar pattern (data not shown). At e12.5, neither alkaline phosphatase nor *Runx2* expression demonstrated the pattern of the future dentary (data not shown).

Expression of T*gf-β 1, 2* & *3* in and around the developing dentary

Tgf-β1 is expressed through out the dentary as it starts to ossify (e13.5-e15.5, figure 2). In contrast, *Tgf-β 2* and *3* are expressed in the surrounding mesenchyme. From e13.5 to e15.5, *Tgf-β 2* is expressed specifically medially and posterior to the angular process. When viewed in sagittal section, this region extends posteriorly from the angular process in a triangular shape. The localisation of *Tgf-β 2* to the tissue around the angular process mirrors the phenotype of the knockout mouse, where the angular is lost (Sanford et al., 1997)

Tgfbr2 wnt1-cre fl/fl phenotype

It has been previously reported that the conditional loss of *Tgfbr2* in neural crest derived tissues results in a hypoplastic mandible, including the loss of the angular process and secondary cartilages (Ito et al., 2003; Oka et al., 2007). This previous work demonstrated the phenotype of the dentary bone by alician blue/alizarin red skeletal preparation at e16.5, by which time the dentary is well developed. To investigate this phenotype further, and to investigate how this defect arises, we carried

out histological analysis of the dentary in the *Tgfbr2 wnt1-cre fl/fl* conditional knockout at earlier stages to that which has been done before: from e13.5 to e16.5. At e14.5, the patterning of the ossified dentary of the *fl/fl* mouse does not differ greatly from that of the wild type littermates in terms of the presence or absence of the posterior dentary processes (figure 3). This is also reflected at e13.5, where he pattern of the expression of *Runx2* in the non-ossified dentary does not differ between wild type and *fl/fl* littermates. At e15.5 the dentary of the *fl/fl* mouse is small compared to wildtype littermates, with no secondary cartilages and reduced bony processes. The angular process is particularly reduced relative to the coronoid and condylar processes. This is even more marked at e16.5, with the angular process reduced to a small spur on the condylar process, which is itself much reduced. At no stage were mature cartilage cells observed on the proximal mandibular processes. The submandibular salivary glands are elevated to the level of the mandible, although it is unclear whether this is due to the reduced mandible or some other patterning defect.

In addition to the loss of hard tissue, there appears to be a loss of some soft tissues. This includes the population of cells that express *Tgf-β 2* around the angular process. *In situ* hybridisation suggests that *Scx*, which is expresses in the *Tgf-β 2* expressing cells around the angular process in the wild type littler mates, is not expressed in the proximal mandible whereas there is a strong expression in more proximal regions (figure 4). The mandibular muscles also appear to be diminished, and their attachment to the dentary is disrupted or absent (figure 3D).

Cultures – timed inhibition of secondary cartilage induction and maintenance.

In order to recapitulate the phenotype of the *Tgfbr2* conditional knock out, and to determine the stages at which Tgf-β signalling is critical for secondary cartilage development, explant cultures of mouse half mandibles were carried out in the manner previously reported by Glasstone (Glasstone, 1968; Glasstone, 1971). Initially, explant cultures were carried out to confirm the ability of this system to develop secondary cartilages. Following culture in BGJb medium, 10%FBS, 100μg/ml ascorbic acid for 4 days, and staining with alcian blue/alizarin red, e15.5 mouse mandibular explants (n=42) show both an increase in the condylar process secondary cartilage in 93% of explants, and development of an angular secondary

cartilage in 60%. A similar pattern of secondary cartilage induction was observed with explants cultured e14.5 mandible explants. In each case, the newly forming cartilage developed as a sesamoid, distinct from the ossified dentary (figure 5A).

Having established our culture conditions, the experiments were repeated using an inhibitor of the downstream signalling of Tgf-βs. SB431542, a small molecule inhibitor of the type I Tgf-β receptors Alk 4, 5 and 7, was chosen as these receptors are the partners of Tgfbr2 in the Tgf-β signalling cascade(Inman et al., 2002; Kitisin et al., 2007). The cultures were carried out at e13.5, e14.5 and e15.5. At e13.5 (data not shown) and e14.5 no secondary cartilage is observed following culture, while at E15.5 the condylar secondary cartilage is clearly visible after alcian blue staining. Half of the head was cultured with inhibitor, while the other half was cultured in control medium, and the pair was compared for alteration in the cartilage pattern after 4 days. Neither e14.5, nor e15.5 explants develop secondary cartilages visible by alcian blue staining in the presence of the inhibitor. Loss of secondary cartilage development in e14.5 explants suggests that Tgf-β signalling is vital in the initiation of secondary cartilages, The already established condylar cartilage of e15.5 explants disappeared after 4 days in culture, indicating the necessity of Tgf-β signalling in maintenance of secondary cartilages. The control sides, however, developed well-formed secondary cartilage, as observed before (Figure 5)

Co-localisation of *Tgf-β 2* and the connective tissue marker *Scleraxis* around the developing angular

Tgf-β2 was shown to have strong expression around the developing angular process in a dense triangular shaped structure visible using histological stains. In order to help characterise this tissue, which might be acting upon the angular process primordium, *in situ* hybridisation for *scleraxis* (*Scx*) and *Myf5* mRNA expression was carried out. *Scx* has been demonstrated to act as a marker for tendon and ligaments, whilst *Myf5* is an early muscle marker.

From e13.5, before the dentary has ossified, *Scx* is expresses around the future sites of muscle attachment of the presumptive dentary at the coronoid and angular process (figure 6). The expression of *Scx*, when observed in sagittal section, takes the form of a thin band along the border of the future bone, it is also strongly co-expressed with *Tgf-β2* in the extended region posterior and medial to the future angular process.

Expression of *Scx* does not however extend as medially as *Tgf-β2*. At this stage, *Myf5* is weakly expressed in the developing extraocular and facial muscles, where as by e15.5 expression is strong in these tissues. At the site of attachment for the temporalis muscle at the coronoid process, there is a close association of *Myf5* expression and the expression of *Scx* at the interface of the muscle with the bone. However, there is no such relationship between *Scx* and *Myf5* in the region around the angular process in which *Scx* shares its expression domain with *Tgf-β2*.

The dense triangular shaped tissue around the developing angular process, which co-expresses *Scx* and *Tgf-β2* mRNA in the wild type mouse (figure 3C asterisk), was absent in the *Tgfbr2 wnt1-cre fl/fl* the conditional knockout.

Tgf-β signalling induces the expression of *Scleraxis* and inhibition of Tgf-β signalling results in a loss of endogenous *Scleraxis* expression.

Due to the co-expression of *Tgf-β2* and *Scx*, we hypothesised that there is a relationship between the two. To test this, e13.5 whole mandibular arch explants were treated with beads soaked in either Tgf-β 1 (n=3), Tgf-β 2 (n=6) or BSA (n=3). Beads were implanted into the mesenchyme around the forming Meckel's cartilage and then the explants were cultured for 24 hours in serum free DMEM medium. Following whole mount *in situ* hybridisation staining for the expression of *Scx* mRNA, it was found that, in all explants Tgf-β 2 was able to induce *Scx* expression in the mandibular arch. This is true for the regions around the endogenous *Scx* domain, and areas away from it (figure 7). In addition, all explants cultured in the presence of Tgf-β 1 beads showed induction of Scx outside of endogenous regions (n=3). There was no evidence that *Scx* was up regulated in the presence of BSA soaked beads (n=6). Furthermore, when cultured in the presence of SB431542 endogenous expression of *Scx* in the mesenchyme around the developing dentary is down regulated in explants of e13.5 – e15.5 mandibular arches (figure 8).

Discussion

The dentary anlage is patterned before ossification, and this appears to be independent of Tgf-β signalling.

Expression of *Runx2* and alkaline phosphatase in the mouse dentary anlage at e13.5 indicates that the dentary has the basic adult bone pattern, with three proximal processes, predetermined before ossification. The work here largely corroborates the earlier work of Miyake and colleagues who investigated the expression of alkaline phosphatase in the developing head (Miyake et al., 1997). However, in this study the coronoid the proximal processes is first described at Theiler's stage 23, which, corresponds to around e15 in the mouse strain used (C57BL/6), where as we demonstrate the pattering to be present at e13.5, which corresponds to Theiler's stages 21-22. This discrepancy may be due to a number or reasons, including the fact that this study and our own used different mouse lines (C57BL/6 as opposed to CD1), however it is more likely that as this study used 3D reconstructions of frontal sections the morphology of the proximal dentary was simple not faithfully maintained. Additionally, Miyake et al demonstrated suggest that development of the secondary cartilages occurs around stage 24, a similar stage to that which we suggest, although no difference is the emergence of the condylar and angular processes are reported.

In *Tgfbr2 wnt1-cre fl/fl mice*, which develop with a hypoplastic mandible including an absent angular process, expression of *Runx2* in the dentary anlage is by and large normal and displays three proximal processes. This suggests that the early patterning of the membranous dentary is independent of Tgf-β signalling. Tgf-β signalling, however, was crucial for the initiation and maintenance of the secondary cartilages.

The secondary cartilages of the condylar and angular processes are first observed at different times

Previous studies suggest that the condylar process is initiated at e16.5 with the development of the secondary cartilage (Livne and Silbermann, 1990). However, our data shows the condylar process is visible with alcian blue staining at e15.5, while the angular process is visible at e16.5. Furthermore, initiation of the secondary cartilages must occur sometime before the appearance of alcian blue positive tissue. Shibata and colleagues have shown that Sox9, an early marker of chondrogenesis, is expressed in

the condylar anlage as early as e14 (Shibata et al., 2006), and so cartilage-inducing signals must have acted upon these cells around this time. This all suggests that researcher must look at least at e15.5, and probably as early as e13.5, when investigating the initiation of the secondary cartilages of the mouse dentary proximal processes.

Essential role for Tgf-β signalling in initiation and maintenance of secondary cartilages

In cultures treated with a Tgf-β signalling inhibitor the secondary cartilages failed to be induced, or if already formed failed to be maintained. Tgf-β signalling is therefore required throughout early secondary cartilage development.

The action of Tgf-β on cartilage development is not clear. In organ cultures of developing mouse metatarsal bone (which, in contrast to the mandible, form by endochondral ossification) Tgf-β 1 has been shown to inhibit hypertrophic differentiation and chondrocyte proliferation (Alvarez et al., 2001), and Tgf-β 2 has been shown to mediate in the inhibitory effect of Indian hedgehog (Ihh) on hypotrophic differentiation and chondrocyte proliferation(Alvarez et al., 2002). When injected in to the periosteum of the femur, Tgf-β 2 can induce the development of connective tissue, which later leads to the development of cartilage (Joyce et al., 1990). Both positive and negative roles have therefore been assigned to Tgf-β. In the secondary cartilages of the dentary, however, the effect of Tgf-β was purely as a positive influence on cartilage development.

In our experiments, we used a general Tgf-β signalling inhibitor to mimic the effect of knockout of the *Tgfbr2*. In this case all three Tgf-βs would have been inhibited (Tgf-β 1,2,3). Knockout mice have been made for each of these ligands. As previously mentioned *Tgf-β2 -/-* have no angular process but are in possession of a secondary cartilage at the site of the process. There is, however, no mandibular defect in the *Tgf-β1 -/-* and *Tgf-β3 -/-* mice (Sanford et al., 1997). Secondary cartilages thus form in each of the three knockouts indicating that the three ligands can compensate for the loss of each other with respect to induction of secondary cartilage.

Mechanical force is important for mandibular patterning, and Tgf-β signalling is involved in tendon development.

Loss of secondary cartilages in the *Tgfbr2* conditional mutant may well play a major role in the later failure of the processes of the dentary to extend. However, secondary cartilages are not necessary for the three-pronged pattern of the proximal dentary. In the conditional knockouts of the BMP type I receptor, *Alk2*, secondary cartilages are absent but the three processes of the dentary are still in evidence, though slightly reduced in size (Dudas et al., 2004). In a similar vein, in the *Tgf-β 2* knockout, the secondary cartilages are initiated as normal but the angular process is still lost. The formation of the angular is therefore not only reliant on the formation of secondary cartilage for its extension and development into a major muscle attachment site. *Tgf-β 2* is expressed around the developing dentary with high levels associated with a triangle of cells under the angular process.

This group of cells also co-expresses *Scleraxis*, a tendon and ligament marker (Liu et al., 1996; Murchison et al., 2007). Previous studies have suggest that *Scx* is induced by Fgf8 (Brent and Tabin, 2004; Manfroid et al., 2006), however, we show that Tgf-β signalling can induce *scleraxis* expression also, and that Tgf-β signalling can induce *scleraxis* expression also, and that inhibition of Tgf-β signalling can interrupt *Scx* expression in the muscle attachment sites dentary bone. Whilst earlier cell culture studies demonstrated an upregulation of *Scx* by Tgf-β treatment in a dose dependant manner (Brent et al., 2003; Brent and Tabin, 2004; Schweitzer et al., 2001), we have demonstrated this effect in explant culture. This indicates an important role for mechanical force in shaping the dentary, in particular the angular process. In the *Tgfbr2* conditional knockout, this triangular group of cells expressing *Scleraxis* and *Tgf-β 2* is lost as is the expression of *Scx in this region*, and this may have resulted in a loss of muscle attachment, and so a loss of mechanical stimulation. Contradictorily, a recent study by Murchison and co workers on the *Scx* -/- mice do not report and loss of the angular process, or indeed any other mandibular phenotype (Murchison et al., 2007). However, in this study it is established that although Scx is a good marker for all tendons, surprisingly the loss of Scx does not result in a loss of all tendons, only the intermuscular tendons and of the tendons responsible for transmitting musculoskeletal force in the limbs, tail and trunk, and there is no effect in those

tendons which anchor muscles to skeletal elements such as the dentary. This study demonstrates that Scx is not a master controller of tendon development, and suggests that there is some as yet unknown factor inducing those tendons unaffected by *Scx* loss.

Rot-Nikcevic and co-workers report that mice lacking both *Myf5* and *MyoD*, and hence lacking any muscle, develop a reduced and immature condylar cartilage, but no angular cartilage or process (Rot-Nikcevic et al., 2007). The pattern of the dentary of the *Myf5 -/-; MyoD -/-* mouse, therefore, resembles very well that of the *Tgfbr2 wnt1-cre fl/fl* conditional knock out. Neither has an angular process, and the other processes are hypoplastic and the secondary cartilage is absent from the angular and reduced in the condylar. This strongly suggests that mechanical forces are in fact important in the mandibular phenotype of the *Tgfbr2 wnt1-cre fl/fl* mouse.

Mechanical force and secondary cartilage formation

In our cultures, the dentary anlage formed secondary cartilages in the absence of mechanical stimulation, agreeing with previous experiments (Glasstone, 1968; Glasstone, 1971). The initiation of secondary cartilage is thus independent of muscle. These cartilages, however, did not mature and undergo secondary ossification. This failure in secondary ossification is probably due to the lack of mechanical force, which is known to be important for the maturation of secondary cartilages (Habib et al., 2005; Hall and Herring, 1990; Herring and Lakars, 1982). It has been demonstrated that the culture of perinatal mandibular condyles of in the absence of mechanical stimulation will result in a loss of the characteristic features of mature cartilage, and an increase hypertrophic chondrocytes and deposition of bone (Silbermann et al., 1987). When condylar explants are cultures with functional loading, mimicking the mechanical stimulation of muscle action, or by electrical stimulation of the attached muscles, the increase in bone production is not seen, and the cartilage is more like that seen *in vivo* (Kantomaa and Hall, 1988; Pirttiniemi and Kantomaa, 1996) The independence of mechanical force and secondary cartilage initiation indicated in the cultures, appears to support the phenotype of the *Myf5/MyoD* double knockout condylar process, but seems at odds with the lack of angular cartilage. It is possible, however, that secondary cartilages may have initiated

in the muscle mutant but failed to be maintained due to loss of muscle activity. Alternatively, is if possible that the cartilage of the condylar process is different to that of the angular.

The proximal processes of the mammalian dentary are differently affected by morphogenic signals.

The data presented here suggests a difference in the action of mechanical and chemical signals on the different dentary processes. Of the three proximal processes, the angular process is the most severely affected by the conditional loss of Tgf-β signalling, and by the loss of muscle development (Rot-Nikcevic et al., 2007). It also appears that there is a greater association between *Tgf-β* and *Scx* at the angular process, suggesting that force transmitted through tendon and ligaments may have a greater affect on this process, compared to the coronoid and condylar process. In contrast, the perseverance of the condylar cartilage in *Myf5/MyoD* mutant, and conditional *Tgfbr2* mutant, suggests that, due to it's importance as the site of articulation with the cranial base, the condylar is likely to be independent of mechanical forces of muscular loading, which may affect the other processes, but may be the consequence of chemical signalling acting along side forces generated by the growth and apposition of tissues. The coronoid process is independently lost in a number of knockout mice, such as *Dlx5* and *Pax9* mutants (Peters et al.,1998; Depew et al. 1999). The development of this process is thus likely to be controlled by factors that do not influence the other processes. This independence of the three processes allows them to change morphology in relative isolation, allowing for a wide variation in shape and size in different mammals.

To summarise, we have shown here that the three-processed pattern of the mammalian dentary is determined prior too embryonic day 13.5, at least 24 hours before the ossification of the bone. Furthermore, we show that this pattern is maintained at this stage in the *Tgfbr2 wnt1-cre* mouse, which is lacking proximal mandibular processes later in development. We show that Tgf-β signalling is essential for initiation and maintenance of secondary cartilages in culture, indicating that the different Tgf-β ligands are able to compensate for each other's loss in knockout mice. Loss of the angular process in the conditional *Tgfbr2 knockout* may be in part due to a

combination of loss of secondary cartilage formation and loss of mechanical force. The region around the angular that expresses *Tgf-β 2*, also expresses the tendon marker *Scleraxis*, which can be induced by Tgf-β signalling. This region is lost in the conditional knockout, resulting in a failure of muscle attachment. We support the notion that that condylar process is under additional developmental signals due to its importance as the site of attachment to the cranial base compared to the coronoid and angular processes. Finally, whilst it is apparent that the actions of muscles and other mechanical forces are important for the differentiation of secondary cartilages into the bones of the processes, and for the development of the angular process in particular, they may not be important for the initial induction of mouse secondary cartilages. The relationship between mechanical force, chemical morphogens and other factors in development is complex and requires further study.

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Figures.

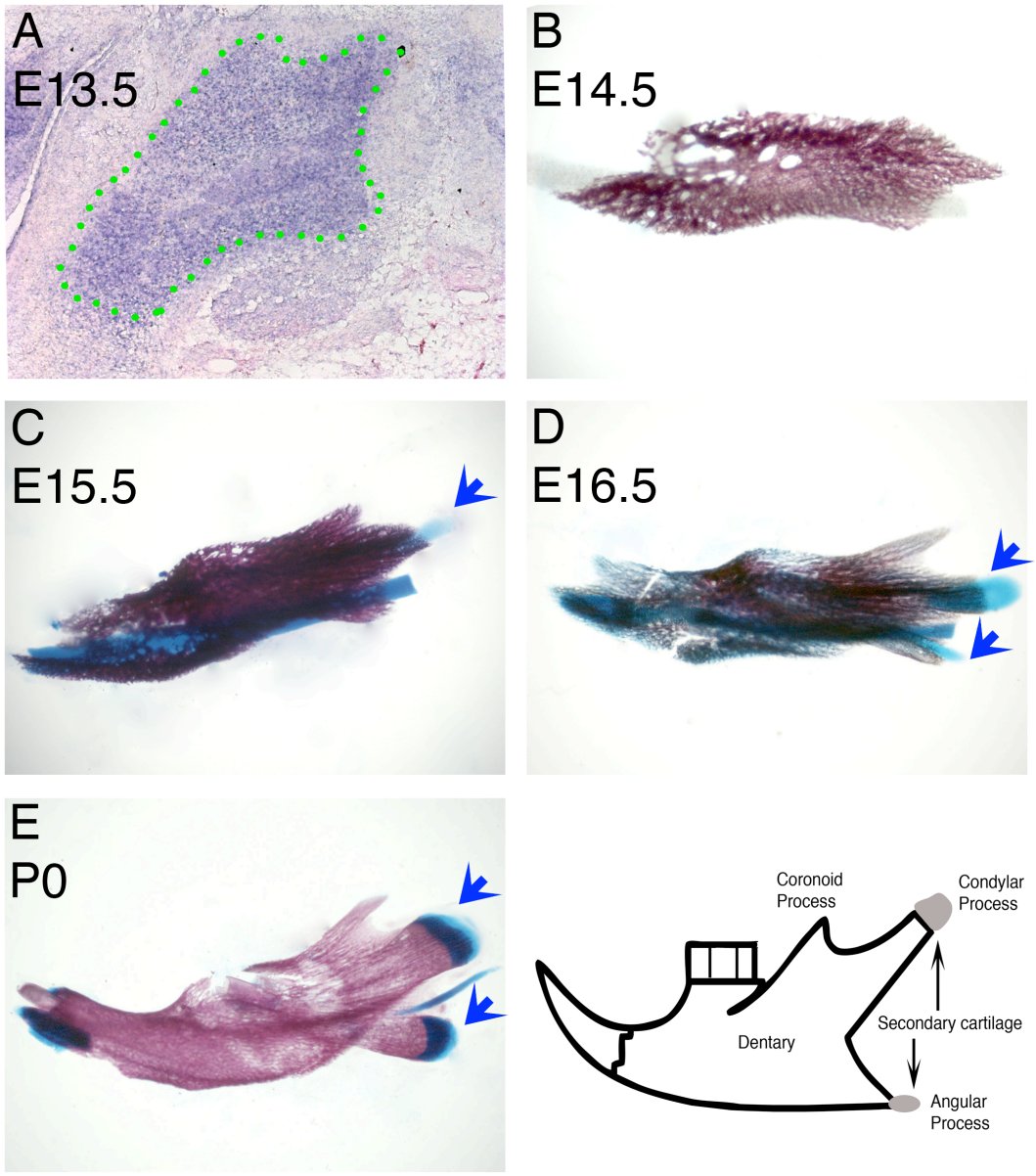

Figure 1: Development of the articular portion of the mouse mandible; A. Parasagittal section through the first branchial arch of an e13.5 mouse showing expression of the early bone marker *Runx2* by Dig labelled *in situ* hybridisation. Outline shows the patterning of condensing mesenchyme in the three processes of the mammalian mandible; B-E. Alizarin red/ alcian blue skeletal preparations of embryonic mouse dentaries between e14.5 and p0. (B. e14.5, C. e15.5, D. e16.5, E. p0) Bone is stained in red, cartilage is stained blue. Red arrows indicate the presence mandibular processes with no secondary cartilage, Blue arrows indicate processes capped with secondary cartilage. F. Schematic of adult mouse mandible.

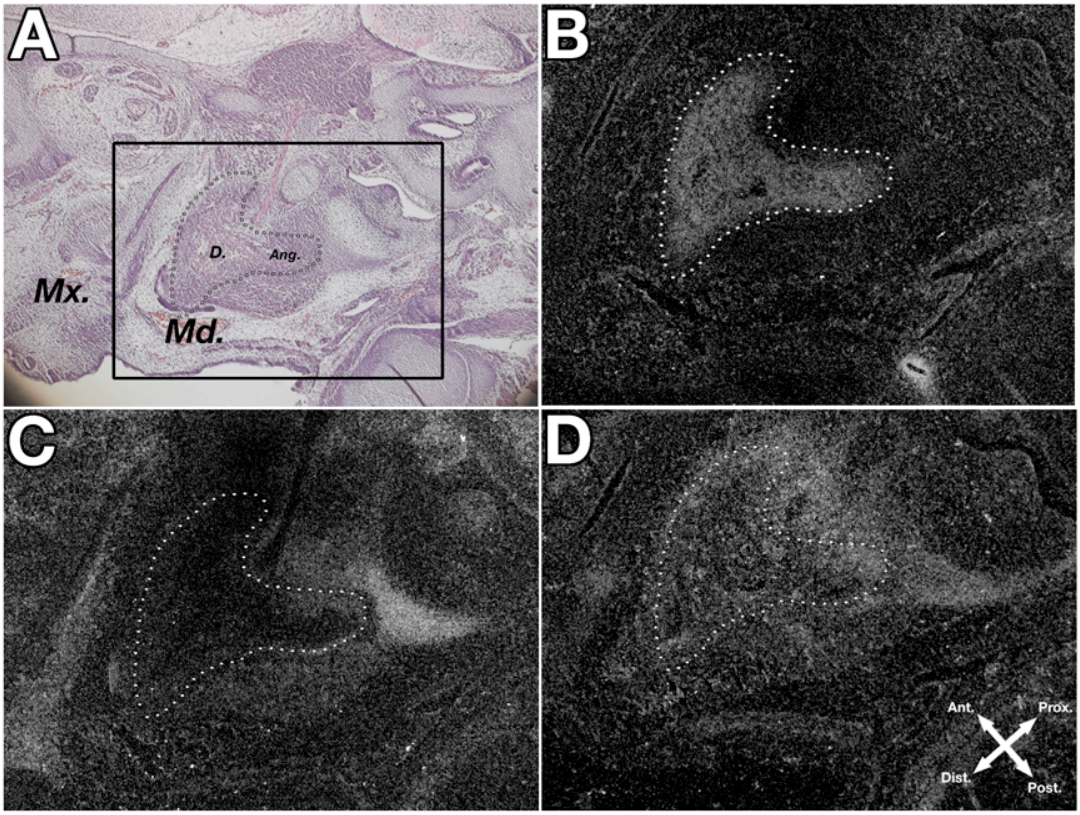

Figure 2: Expression of the three isoforms of transforming growth factor β in serial sagittal sections of a mouse mandible at e14.5; A. Haematoxylin and eosin stain. Dotted line highlights developing dentary bone. *Mx*. – Maxilla, *Md*. – Mandible, *D*. – Dentary, *Ang*. – Angular process of dentary; B-D. Expression by radioactive *in situ* hybridisation of *Tgf-β1* (B), *Tgf-β2* (C) and *Tgf-β3* (D). *Tgf-β1* is highly expressed in bone of dentary, *Tgf-β3* is generally expressed in the undifferentiated mesenchyme of the mandible, *Tgf-β1* is expressed in around the developing dentary.

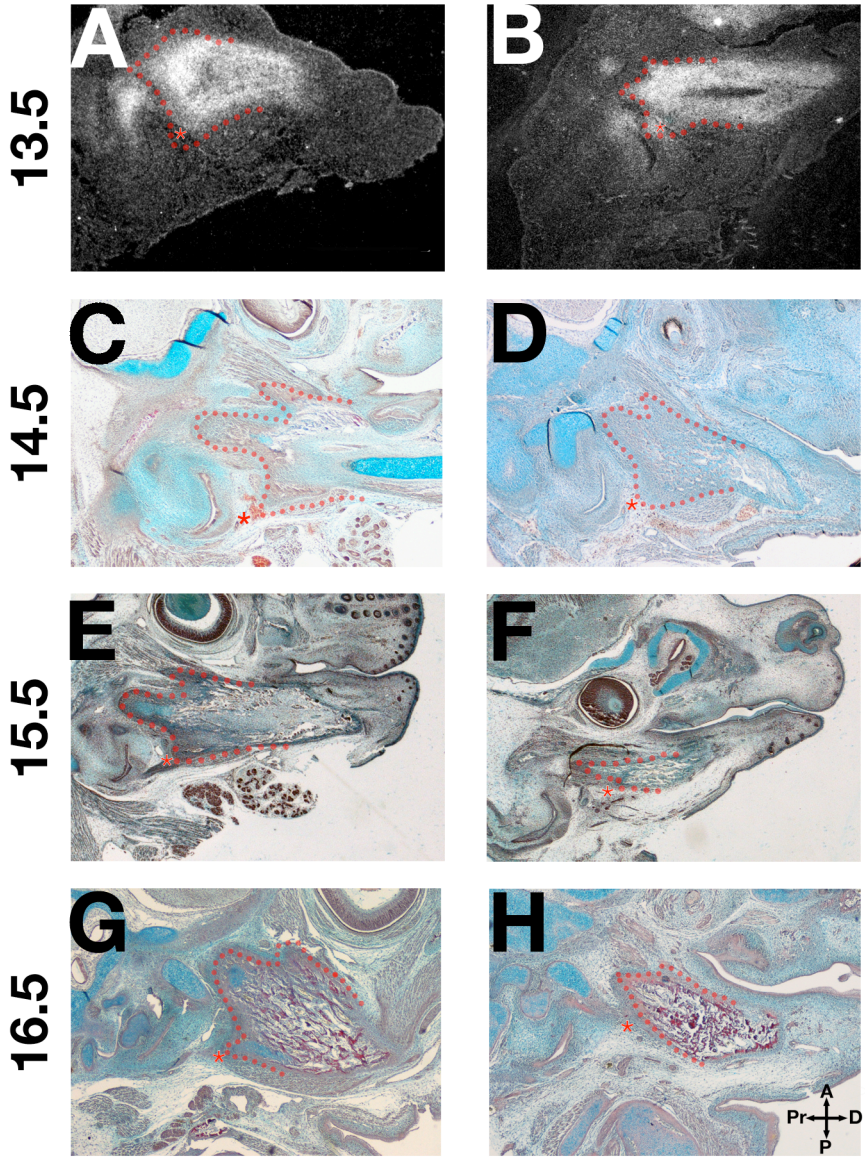

Figure 3: Comparison of the development of the dentary of *Tgfbr2 wnt1-cre fl/fl* conditional knockout mice and wild type littermates: A-B, Expression by radioactive *in situ* hybridisation of *Runx2* in sagittal sections of the dentary anlage of wildtype (A) and *Tgfbr2 wnt1-cre fl/fl* (B) mice. The basic three processed pattern of the dentary is seen in both the wildtype and conditional knockout; C-G, Alician blue stain of the dentary of wildtype (C, E, G) and *Tgfbr2 wnt1-cre fl/fl* (D, F, H) mice. By e15.5 (F) angular processes is reduced to a spur on the inferior aspect of the condylar process in the conditional knockout, and no secondary cartilage is observed by alcian blue staining. Red asterisks indicate position of the angular process.

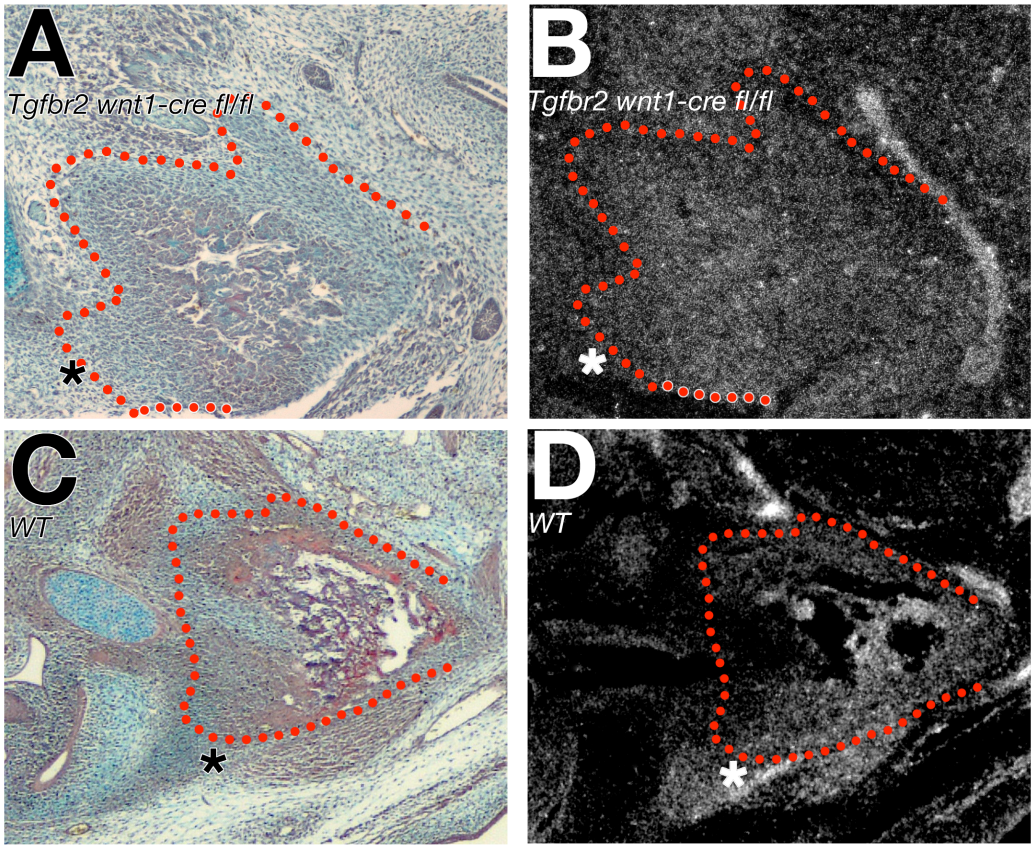

Figure 4: Expression of Scx in the dentary of *Tgfbr2 wnt1-cre fl/fl.* A. Sirrius red / alician blue stain of sagittal section of the dentary if e14.5 *Tgfbr2 wnt1-cre fl/fl*. B. Expression of Scx in sagittal section of the dentary if e14.5 *Tgfbr2 wnt1-cre fl/fl.*

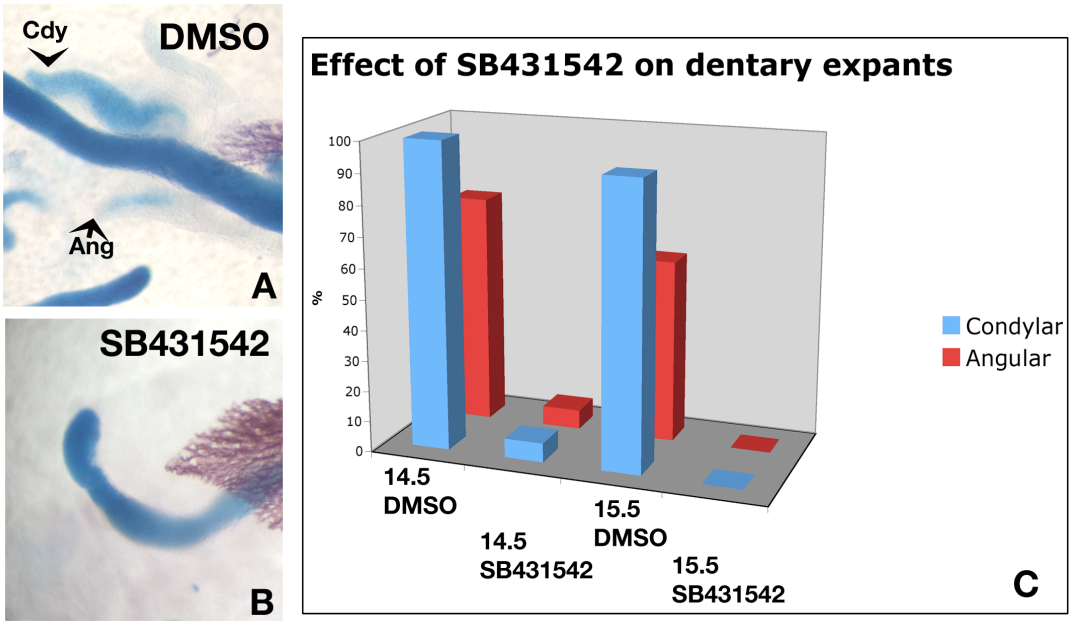

Figure 5: Effect of SB431542, a small molecule inhibitor of Type I receptor targets of Tgfbr2 on secondary cartilage in e14.5 +5 day mandibular explant cultures in serum free medium. A. Control e14.5 +5 day explants cultured with 10µl DMSO per ml medium stained with alcian blue and alizarin red. Cdy. Condylar secondary cartilage, Ang. Angular secondary cartilage. B. e14.5 +5 day explants cultured with 100mM SB431542, stained with alcian blue and alizarin red.

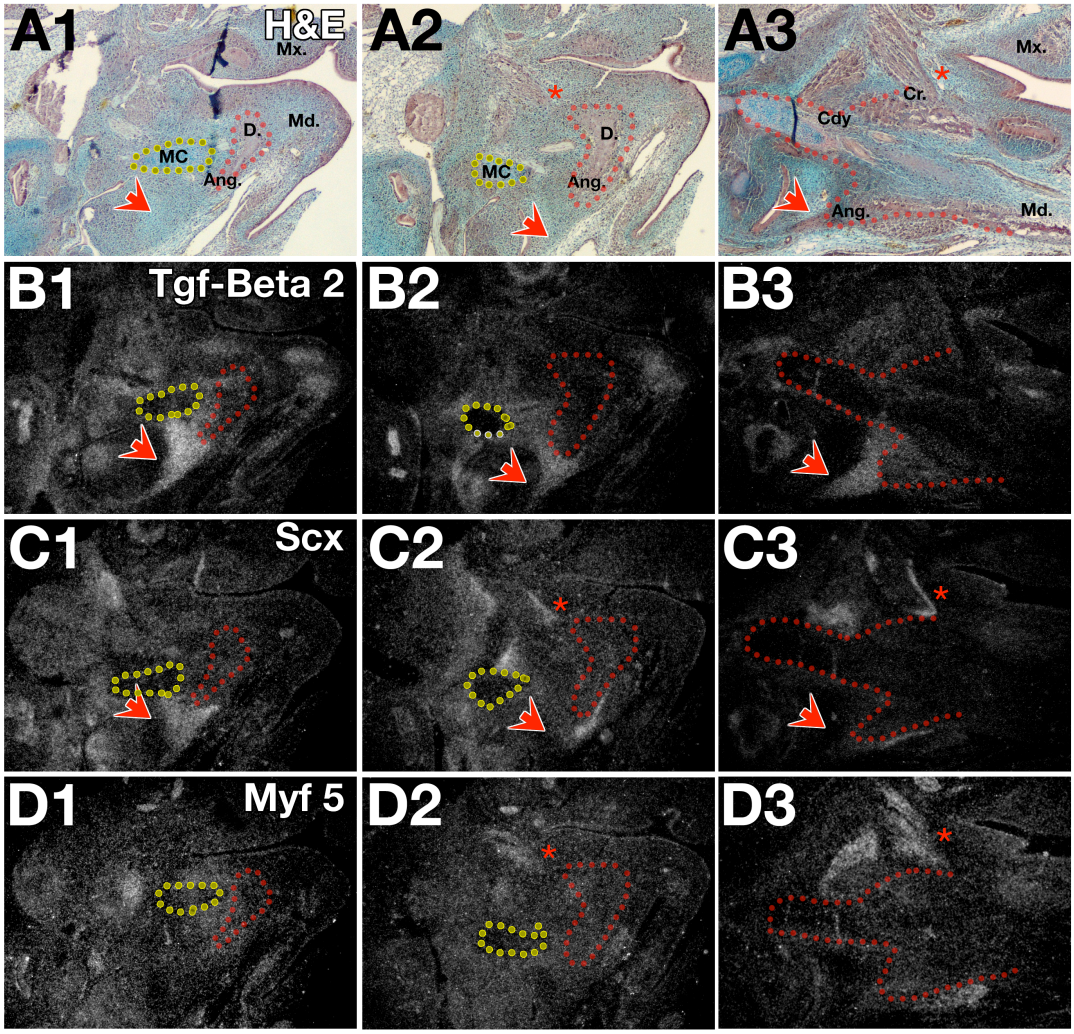

Figure 6: Expression of *Tgf-β 2*, tendon marker *Scx* and muscle marker *Myf 5* in the developing articular region of the mouse mandible at e13.5 and e15.5: A1-D1 and A2-D2, serial sagittal sections of e13.5 mouse mandibles at medial (A1-D1) and lateral (A2-D2) positions through the mandible; A3-D3, serial sections through the dentary of e15.5 mouse mandible; Arrowhead indicates dense triangle of *Tgf-β2* expression cells around the angular process. Red asterisks indicate site of attachment of m. temporalis on the coronoid process. A1-3. Alcian blue staining. Mx. Maxilla, Md. Mandible, D. Dentary, Cdy. Condylar, Ang. Angular, Cr. Coronoid, MC. Meckel's cartilage. B1-3, Radioactive *in situ* hybridisation for Tgf-β 2. Expression around the angular process at e13.5 is found at medial and lateral positions. C1-3, Radioactive *in situ* hybridisation for *Scx*. At e13.5 *Scx* is co-expressed with *Tgf-β2* around the angular process at medial positions (C1) but not laterally (C2), nor at e15.5 (C3). *Scx* is expressed at the muscle attachment sites on the coronoid and angular process from e13.5 (C2-3). D1-3, Radioactive *in situ* hybridisation for *Myf5*.

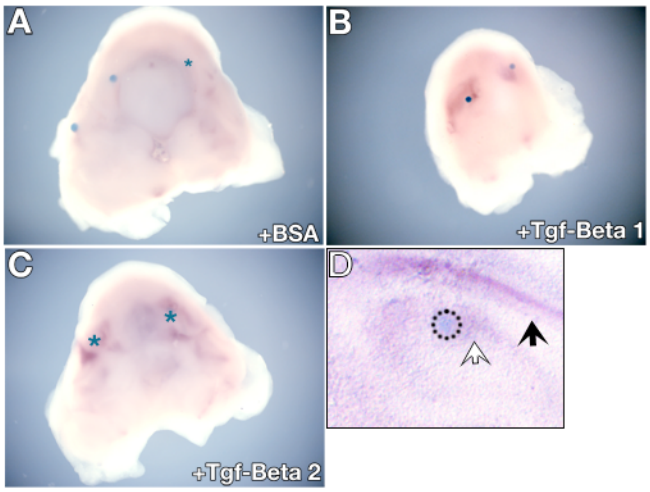

Figure 7: Induction of the expression of *Scx* in mandibular explants by Tgf-β protein. Ectopic expression of *Scx* is not induced when mandibular arches are cultures with beads loaded with BSA (A), unlike in those explants cultured with Tgf-β1 (B) or Tgf-β2 (C, D) beads. Black arrow shows endogenous *Scx* expression, white arrow shows ectopic *Scx* expression.

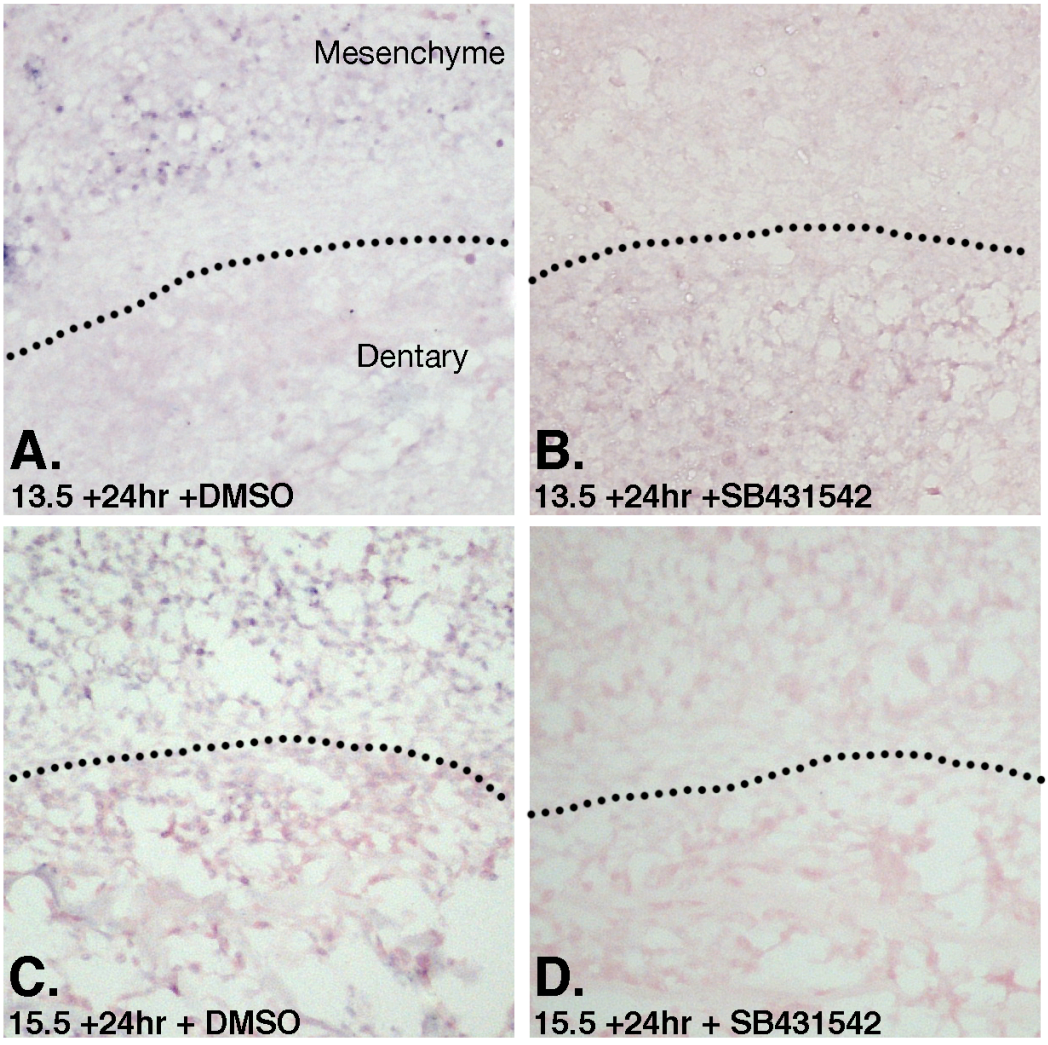

Figure 8: The inhibition of expression of *Scx* in the mandibular mesenchyme upon chemical inhibition of Tgf-β signalling. *In situ* hybridisation for the expression of *Scx* mRNA on sections of e13.5 (A, B) and e15.5 (C,D). Culturing in the presence of Tgf-β signalling inhibitor SB431542 (B, D) results in loss of the expression of *Scx* in the mesenchyme of the muscle attachment sites on the developing dentary bone (A, C).